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Meeting Report

Antibodies targeting G protein-coupled receptors: Recent advances and therapeutic challenges

Mohammed Akli Ayoub[1,2,3,*], Pascale Crépieux[1], Markus Koglin[4], Marc Parmentier[5], Jean-Philippe Pin[6,7], Anne Poupon[1], Eric Reiter[1], Martine Smit[8], Jan Steyaert[9], Hervé Watier[10,11], and Trevor Wilkinson[12].

[1]PRC, INRA, CNRS, Université François-Rabelais de Tours, 37380 Nouzilly, France

[2]LE STUDIUM® Loire Valley Institute for Advanced Studies, 45000 Orléans, France

[3]Biology Department, College of Science, United Arab Emirates University, PO Box 15551 Al Ain, United Arab Emirates

[4]Heptares Therapeutics Ltd, BioPark, Broadwater Road, Welwyn Garden City, Hertfordshire AL7 3AX, UK

[5]Institut de Recherche Interdisciplinaire en Biologie Humaine et Moléculaire, Université Libre de Bruxelles, B-1070 Brussels, Belgium; Welbio, Université Libre de Bruxelles, B-1070 Brussels, Belgium

[6]Institut de Génomique Fonctionnelle (IGF), Université de Montpellier, CNRS UMR5203, 34094 Montpellier, France

[7]INSERM U1091, 34094 Montpellier, France

[8]Amsterdam Institute for Molecules Medicines and Systems (AIMMS), Division of Medicinal Chemistry, Vrije Universiteit, Amsterdam, The Netherlands

[9]Structural Biology Brussels, Vrije Universiteit Brussels, B-1050 Brussels, Belgium; Structural Biology Research Center, Vlaams Instituut voor Biotechnologie, B-1050 Brussels, Belgium

[10]Université François-Rabelais de Tours, CNRS, UMR 7292, Tours, France

[11]Laboratoire d'Immunologie, CHRU de Tours, Tours, France

[12]Antibody Discovery and Protein Engineering, MedImmune, Milstein Building, Granta Park, Cambridge CB21 6GH, UK

CONTACT: Mohammed Akli Ayoub. *Email: mayoub@uaeu.ac.ae*, Biology,United Arab Emirates University,Building F1,Office 190,Al Ain,Abu Dhabi,United Arab Emirates,15551

LE STUDIUM Loire Valley Institute for Advanced Studies Conference November 24--25, 2016 - Tours, France

ABSTRACT

Le STUDIUM conference was held November 24--25, 2016 in Tours, France as a satellite workshop of the 5th meeting of the French GDR 3545 on "**G Protein-Coupled Receptors (GPCRs) - From Physiology to Drugs**", which was held in Tours during November 22--24, 2016. The conference gathered speakers from academia and industry considered to be world leaders in the molecular pharmacology and signaling of GPCRs, with a particular interest in the development of therapeutic GPCR antibodies (Abs). The main topics were new advances and challenges in the development of antibodies targeting GPCRs and their potential applications to the study of the structure and function of GPCRs, as well as their implication in physiology and pathophysiology. The conference included two sessions, with the first dedicated to the recent advances in methodological strategies used for GPCR immunization using thermo-stabilized and purified GPCRs, and the development of various formats of Abs such as monoclonal IgG, single-chain variable fragments and nanobodies (Nbs) by *in vitro* and *in silico* approaches. The second session focused on GPCR Nbs as a "hot" field of research on GPCRs. This session started with discussion of the pioneering Nbs developed against GPCRs and their application to structural studies, then transitioned to talks on original *ex vivo* and *in vivo* studies on GPCR-selective Nbs showing promising therapeutic applications of Nbs in important physiological systems, such as the central nervous and the immune systems, as well as in cancer. The conference ended with the consensus

that Abs and especially Nbs are opening a new era of research on GPCR structure, pharmacology and pathophysiology.

KEYWORDS

antibody, nanobody, biopharmaceuticals, GPCR, G protein, β-arrestin, phage display.

Thursday November 24, 2016

Overview on the "Biopharmaceuticals" Program ARD2020 Launched by Centre Val de Loire Region (France)

Prof. Hervé Watier

Hervé Watier (Université François-Rabelais de Tours, CNRS) opened the meeting by presenting the "Ambition Research and Development 2020 Biopharmaceuticals" program launched by the region Centre Val de Loire in France. Having first defined the term "biopharmaceuticals" (~therapeutic proteins) [1] and the major role played by monoclonal antibodies (mAbs) to sustain the extraordinary dynamic of this pharmaceutical sector, he explained that France lags behind, and particularly the region Centre Val de Loire, despite the importance of its pharmaceutical industry, which mainly manufacturers chemical drugs). To change the situation and to favor diversification, the French government and the regional council have both relied on academic research, by providing substantial funding to the MAbImprove LabEx over the 2011--2019 period [2] and the "Ambition Research and Development 2020 Biopharmaceuticals" program over the 2014--2019 period. Both programs are expected to generate new discoveries and inventions, as well as new know-how and technologies, and to transfer them to the industrial sector (e.g., start-up, biotech and pharmaceutical companies, contract research and manufacturing organizations). Some successes have already been achieved, despite the fact that these ambitious national and regional policies must be evaluated over the long-term. Beside the universities and the public research institutes, several regional actors participate in the regional program, such as Polepharma, a pharmaceutical cluster, the IMT group, which is in charge of the Bio^3 Institute for bioproduction training, and Le STUDIUM Loire Valley Institute for Advanced Studies, which hires skilled researchers and helps them to organize very focused symposia/conference, with high international visibility, such as the present one organized by Dr. Mohammed Akli Ayoub. The program he has prepared for this

conference fits very well with the first of the four key questions that drive the scientific projects of the MAbImprove LabEx in the field of therapeutic antibodies: How can target activity be modulated through antibody binding? G protein-coupled receptors (GPCRs) are probably the most emblematic field for pharmacological modulation via mAbs.

Using Stabilized GPCRs as Antigens to Generate Functional Antibodies

Dr. Markus Koglin

The first session of the conference on GPCR antibodies (Abs) started with a talk by **Markus Koglin** (Heptares Therapeutics Ltd), who introduced the real context of drug discovery in the field of GPCRs and antibodies. Indeed, despite being one of the largest classes of proteins, the majority of GPCRs remains undrugged, with only ~70 and ~30 GPCRs targeted with small molecules and peptides, respectively. Based on internal triaging of GPCR targets for antibody discovery, around 100 targets provide a clear biological rationale, with 50% of these targets having significant and high clinical value. Over the past decade, substantial progress has been made in generating clinical antibody candidates against a broad variety of targets. Furthermore, advances in structural insights in GPCR function has highlighted the opportunities that highly selective mAbs against GPCR targets could provide to patients.

Despite the progress, only one mAb, anti-CCR4 mogamulizumab, is currently marketed (in Japan), [3,4] and generation of high quality, functionally relevant Abs targeting GPCRs remains a challenge. One of the main obstacles is the generation of homogenous functional antigens in a clinically relevant conformation. GPCRs are expressed as part of the membrane lipid-bilayer of the cell to a relative low level compared to soluble targets. Furthermore, most of the receptor protein is not available as antigen because only the extracellular domains (N-terminus and extracellular loops) can be targeted for antibody drug discovery. These putative epitopes tend to be highly flexible, which reflects the variable conformational nature of GPCRs. Recent progress has been

made to address the main obstacles in generating suitable antigen GPCR material, and the focus has been to overcome low functional expression levels, improving final antigen quality and reducing receptor heterogeneity.

Dr Koglin briefly discussed the advantages of mAbs compared with small molecules as new therapeutics targeting GPCRs, which has previously been described in detailed. [5] Next, he described the approaches used by Heptares Therapeutics for thermostabilization of GPCRs. Examples of immunization with the different StaR GPCRs as antigens and screening of specific and functional mAbs were presented, and he then discussed the stabilized receptor (StaR) generation process. The ideal GPCR antigen format would include a combination of highest purity and activity together with all the relevant post-translational modifications, and the antigen would be present in a single conformation that addresses the target product profile. Such an antigen only exists in approximation of the ideal case scenario because the preparation of purified, high quality GPCR protein is very difficult. Heptares Therapeutics has developed a technology that allows the thermostabilization of GPCRs with increasing stability in short-chain detergents and stability during the purification process, and it also ensures the quality of the purified material over time. [6] The technology utilizes selected single amino acid mutations to introduce or stabilize the receptor to increase high quality receptor protein in a detergent environment. [7] These StaRs can be locked in a conformation derived from the pharmacology of the ligand used in their creation as an iterative process. Increasing thermostability allows the extraction of functionally active protein from the membrane environment to be maximized, as highlighted by increases in maximal radioligand binding with increased mutational load. The process also promotes an increase in receptor homogeneity, as often seen by experimental analysis like fluorescent size-exclusion chromatography (FSEC). Initially labile receptors can be extracted from the membranes in shorter-chain detergents suitable for antibody discovery or crystallization approaches after the stabilization process. [8] Furthermore, stability over time is increased compared to the wild-type receptor as

shown by half-life experiments and by stable and monodisperse FSEC profiles after incubation periods at varying temperatures or even after the receptor has been frozen. For antibody drug discovery approaches, mutations in the extracellular loops or the N-terminus of the receptor can be avoided, but the introduction of mutations needs to be monitored by pharmacological approaches to evaluate the effect on overall receptor pharmacology.

Dr Koglin then discussed construct optimization. The design of constructs suitable for crystallization focuses on the generation of a very stable and very rigid protein to facilitate crystallization, [9] and, to a certain degree, such considerations also apply for the production of GPCRs as immunogens. There are limitations in the construct design because changes of any extracellular domains of the GPCR target are not desirable as such changes may generate antibodies do not recognize the wildtype receptor. The removal of post-translational modifications should be addressed very carefully because they can be essential for correct processing of the GPCR to the cell surface or are directly involved in ligand binding. Changes to the C-terminus of the receptor are considered less disruptive for a GPCR antigen design, but also can affect receptor cell surface expression profiles and may affect the overall conformation of the receptor or receptor internalization. Matrix-based approaches have been used to successfully identify receptor constructs that displayed a monodisperse appearance compared to the full-length receptor without affecting receptor pharmacology as determined by radioligand affinity measurements. Modification of the intracellular loops is widely used for the generation of crystallization constructs because it will remove very flexible regions that can be of substantial length and be prone to degradation when over-expressed. Removal of internalization or degradation signals within the receptor sequence can further improve receptor stability on the cell surface. The introduction of stable fusion partners has been very successful in aiding GPCR crystallization, but it can have a substantial effect on receptor conformation in the extracellular domains of the receptor, and thereby interfere with the generation of a suitable biopharmaceutical. Dr Koglin noted that, in his experience, conformationally sensitive

antibodies have been known to lose the capacity to bind to the receptor extracellular surface upon introduction of a fusion partner within the intracellular loop region highlighting the issue of large fusion partners.

Construct design will facilitate the production of the GPCR protein and can be monitored by functional analysis, but the receptor will still fluctuate in a number of naturally existing conformations (active/inactive and variations of these basic states). To reduce such flexibility, GPCRs are purified and crystallized in the presence of a ligand that confers structural rigidity through interaction with parts of the receptor molecule. The presence of a ligand has been crucial to obtain GPCR structures, but potentially will mask or block amino acids suitable as interaction partners for the desired antibody format. The presence of large ligands or ligands that interact with cell surface-exposed domains of the receptor can therefore be counterproductive for antibody generation. The use of ligands that allow a ternary complex between ligand, receptor and antibody would circumvent the issue. Recently, small allosteric modulators have been identified that bind the receptor away from potential antibody interaction sites and could be of interest in stabilizing a desired receptor conformation during antibody discovery procedures. [10,11]

Dr Koglin provided examples to highlight antibody discovery using in vitro and in vivo approaches. Receptors presented as antigens in detergent micelles can be used by *in vitro* antibody screening approaches, but care has to be taken to choose the right detergent because mild detergents might mask important antigen epitopes due to longer detergent carbon chains. This issue can be overcome either by the reconstitution of the purified material into proteoliposomes [12] or the switch to a shorter chain detergent, but this will not work in most cases for instable GPCRs. Thermostabilization allows the generation of highly purified GPCR proteins stabilized for a selected detergent environment compatible with antibody drug discovery, and they will have been generated in a ligand-free and disease-relevant receptor conformation during the screening process. Such material is considered a suitable antigen format for *in vitro* screening approaches. Furthermore, the

purified receptor protein can support downstream screening and evaluation of antibody hits and candidates (e.g., biophysical measurements, epitope mapping).

The advantages of stable and highly purified protein in a suitable detergent micelle as described above can facilitate *in vitro* screening procedures like display technologies. The StaR protein exhibits extended durability and can be stored or re-used once immobilized. The high degree of purity will reduce false positive/non-specific binders relative to cell/liposome based methods, and allows an increase density of antigen material on the immobilization surface. This has been successfully implemented using the ADRB1 StaR protein in phage display of single-chain variable fragments (scFvs). Specific scFv binders were obtained with affinities between 9--400 nM as analyzed by surface plasmon resonance (SPR) using the purified receptor.

Antibody selections using the Ylanthia® library from MorphoSys generated highly diverse and specific hits. A high hit rate on cell surface expressed wild-type receptor with no detectable background binding indicates high specificity obtained with the purified antigen. A diverse set of unique antibodies were selected and only few recognized linear epitopes. Two selected candidates showed 100% inhibitory functional response in a β-arrestin assay format. Furthermore, an effect seen during many GPCR thermostabilization campaigns is the increase in functional expression level on the cell surface as exemplified by whole cell radioligand saturation binding assay results. This allows the progression of even poorly expressed receptor targets to purification, and ultimately to the initiation of antibody discovery projects. A low-level expressed receptor target was stabilized in an agonist confirmation and purified from mammalian cells in a mild detergent. Receptor profiles using size-exclusion chromatography were unchanged after a freeze/thaw cycle and room temperature incubation steps indicating that the receptor was suitable for phage library screening. The screening strategies included solid phase, solution and bead-based approaches. The primary ELISA screening on StaR and irrelevant antigen led to a selection of 39 IgGs. Twenty of these

purified antibodies were specific for the StaR protein in a direct ELISA format with EC_{50} values ranging from 0.4-115 nM.

The same antigen format can also be used for *in vivo* immunization approaches, [13] but rapid dilution of the protein and the detergent concentration below the critical micelle concentration level *in vivo* needs to be taken into account when choosing the detergent environment. As shown by radioligand binding measurements on purified receptor, the addition of adjuvants commonly used for immunization strategies did not affect receptor integrity for a number of commercially available adjuvants tested. The characteristics of a stabilized GPCR not only allows the immunization with purified protein, but allow it to also be used in a combination approach using either DNA (due to increased inherent expression level of the StaR as well as reduced internalization/signaling & being presented in a single confirmation) or whole cells expressing the StaR (with selection of disease-relevant cellular environment using BacMam technology). A more recent report has utilized a thermostabilized form of the turkey β1-adrenergic receptor (β1AR) as antigen.[13] mAbs were generated by a combination of DNA immunization followed by protein boosting. The immunization strategy implemented in the study identified 5 mAbs from 1000 initial clones that bound specifically to HEK-293T cells transiently expressing β1AR and showed no background binding to control transfections. Epitope binning using SPR analysis with purified β1AR revealed that three different epitopes were targeted by the selected antibody panel. Four of the 5 antibodies stimulated cAMP production with EC_{50} values in the range of 0.5-1.5 nM, but did not reach the same maximal response as observed with the isoprenaline control. The antibody panel was cross-reactive to the human receptor as determined by their ability to stimulate cAMP production of the human receptor. In contrast to cAMP stimulation, mAb1 and mAb3 had no effect in a β-arrestin recruitment assay, indicating a bias towards the G protein signalling pathway. Investigation of the potential mode of action using small molecule controls indicates allosteric modulation of the receptor. mAb3 was selected for its effects in a rat cardiovascular model and the observed tachycardic effect (not seen

with a control IgG1 antibody) is consistent with agonism of β1AR. A possible mode of interaction with the receptor was investigated via FSEC analysis and indicated that receptor dimerization caused by one of the antibodies is in agreement that agonist activity was only observed with the full IgG format, but not with the monomeric Fab format.

Development of Therapeutic Monoclonal Antibodies to G Protein-Coupled Receptors: Opportunities and Challenges

Dr. Trevor Wilkinson

Trevor Wilkinson (MedImmune) described approaches to generate functional mAbs against GPCRs as potential therapeutics. Drug discovery targeting GPCRs, and particularly isolation of functional antibodies against these complex membrane proteins, remains a challenging area. Discovery of such antibodies is technically challenging for several reasons already mentioned, including poor stability of many GPCRs as purified proteins and limited epitope accessibility for some classes of GPCR. Dr. Wilkinson presented two case studies that demonstrated successful isolation of functional antagonist antibodies. In the first case study, antibodies to human CXCR2 were generated by *in vivo* immunization and by *in vitro* phage selection methods. Whole cell immunization of transgenic mice with cells overexpressing CXCR2, followed by screening of hybridomas for antibodies binding selectively to CXCR2 overexpressing cells and subsequent screening for inhibition of IL-8 binding to the receptor led to the isolation of antibody HY29-1. [14] The antibody fully inhibited all CXCR2 agonists tested. In a parallel approach, anti-human CXCR2 antibodies were isolated from naïve phage display libraries by using purified CXCR2 reconstituted in magnetic proteoliposomes to enrich for CXCR2 specific scFv. Unique scFv binders which bound CXCR2 expressing cell lines were converted to IgG format and tested for their ability to inhibit agonist driven calcium responses. Interestingly, the phage display derived antibodies showed ligand dependent differences in functional assays. Epitope competition and mapping experiments

showed that the hybridoma and phage display-derived antibodies recognized distinct epitopes of CXCR2. This study demonstrated the benefits of using different antigen presentation methods to isolate functionally diverse antibodies.

In the second case study, antibodies to human formyl-peptide receptor 1 (FPR1) were isolated by immunization of transgenic mice with HEK cells overexpressing FPR-1. [15] Hybridoma supernatants were screened for binding to cells expressing either human or cynomolgus FPR-1 in order to obtain a species cross-reactive antibody that is desirable to facilitate preclinical safety studies. This approach led to isolation of an antibody, Hy38-1, which bound both human and cynomolgus FPR-1, albeit with weak activity against the cynomolgus receptor. To further improve potency and cynomolgus FPR-1 binding, an extensive antibody optimization campaign was performed using Hy38-1 scFv phage display libraries and selections on cells expressing FPR-1. The optimization campaign involved targeted mutagenesis of all six CDRs followed by CDR recombination and random mutagenesis. This led to antibody Fpr0165 which, when reformatted as an IgG, showed similar apparent affinity to human and cynomolgus FPR-1 and potently inhibited FPR-1-mediated primary neutrophil responses.

In silico Approaches to Develop and Identify New GPCR Antibodies

Dr. Anne Poupon

The development of a therapeutic antibody, from the identification of a target of interest to approval of a safe and efficient drug, is a tedious and uncertain process. Convinced that bioinformatics could facilitate many of the steps along this path, the Biology and Bioinformatics of Signalling Systems (BIOS) group, under the guidance of **Anne Poupon** (Université François-Rabelais de Tours, CNRS, INRA), has put a great deal of effort into the design of such methods. Two of them, MAbTope and MabCross, were presented in the meeting.

Most antibodies are obtained through animal immunization. Once antibodies binding the target have been recovered, they need to be characterized. One important characteristics of an antibody is the epitope it recognizes. The gold standard for epitope determination is the 3D structure. However, not all antibody-antigen complexes can be crystallized, or are suitable for NMR. Experimental methods allowing mapping of the epitope rely either on the hybridization of the antibody with overlapping peptides of the target spotted on a membrane, or on the mass spectrometry determination of target regions protected within the complex. Both methods are expensive and require highly specific expertise and equipment. Moreover, the results are often difficult to interpret, or even misleading. Different prediction methods have been designed, but their accuracy remains questionable for routine use. The BIOS group has developed MAbTope, a method for epitope determination that combines a high accuracy prediction method with experimental validation, allowing the fast and low cost determination of the epitope in all tested cases. The first step uses a docking algorithm inspired by PRIOR1, [16] which was optimized in order to generate 30 docking poses paving the epitope. The second step, based on these 30 conformations, involves the design of four so-called epitope peptides. On a benchmark of 131 antibody-antigen complexes of known 3D structures of the complex and of the isolated target and antibody, at least one of these four peptides belongs to the actual epitope (unpublished data). The third step consists in experimentally measuring the binding of the antibody to these peptides. This method has been successfully applied to two therapeutic antibodies that had unknown epitopes.

Another important step in antibody characterization is the search for alternate targets (also called off-targets) because undesired binding of the antibody to molecules other than the desired target can be the source of side effects. Polyspecificity can be assessed by testing the binding of the antibody to large arrays of proteins (mostly membrane proteins), which nevertheless represent only a small fraction of possible alternate targets. Dr. Anne Poupon presented a new method, MAbCross, which allows the prediction of alternate targets. MAbCross is based on an original

method to evaluate the distance between two antibodies, which allows similarities between antibodies that seem different at the primary sequences level to be found. Here also, the MAbCross method has been validated experimentally: for an initial antibody published by the group of Dr Martine Smit, a secondary target was found, unrelated to the initial one.[17]

In many instances, an antibody with potentially important biological activity cannot be further developed due to one or more issues related to, for example, toxicity, solubility, production yield, or intellectual property. MAbCross provides a suitable opportunity by finding unrelated antibodies that bind to the same epitope as an initial antibody. The BIOS group has demonstrated its ability to find unrelated antibodies that bind the targets of the initial antibodies. Moreover, in the case of modulating antibodies (i.e., antibodies triggering pharmacological effects on the target), data support the idea that the new antibodies can elicit biological activities different from the initial one, thereby generating pharmacological diversity through the interaction with a single epitope.

Friday November 25, 2016

Nanobodies Modulating Chemokine Receptor Function *in vitro* and *in vivo*

Dr. Martine Smit

Martine Smit (Vrije Universiteit Amsterdam) described how Nbs targeting chemokine receptors, belonging to the family of GPCRs, effectively and selectively modulate the function of these receptors both *in vitro* and *in vivo*. Chemokine receptors, which are responsible for the trafficking of leukocytes, are implicated in several pathologies, such as inflammatory diseases, cancer and HIV infection. These receptors are therefore attractive therapeutic targets. [18] Via DNA and whole cell immunization of llamas, phage display and counterselection, the first Nbs targeting the extracellular side of GPCRs, and more specifically chemokine receptors, were identified. The CXCR4-specific Nbs antagonize CXCL12-dependent binding and signalling, inhibit HIV-1

replication *in vitro* and induce stem cell mobilization *in vivo*. [18] CXCL12 binds to CXCR4 via interactions of the N-terminus and the extracellular loop 2 (ECL2). [18] Epitope mapping revealed that the CXCR4 targeting Nbs bind with high affinity and specificity to ECL2. The aspartic acid at position 187, involved in binding of CXCL12, appears to be critical for the CXCR4 targeting Nbs, underlining their competitive behavior. Although human and murine orthologues are highly homologous, variations are found in ECL2, which explains why the identified CXCR4 Nbs do not bind murine CXCR4. Biparatopic CXCR4-Nbs show enhanced affinities and potencies. Moreover, these biparatopic Nbs act as inverse agonists on the constitutively active mutant CXCR4, while the FDA approved CXCR4 antagonist AMD3100 (plerixafor, Mozobil^{TM}) acts a neutral antagonist. [17] In particular, the expression levels of CXCR4 in tumor cells are elevated, which may be associated with increases in basal activity. Hence, the use of inverse agonistic CXCR4 Nbs could be beneficial. Since these biparatopic Nbs bind to distinct and partially overlapping epitopes, these Nbs are suggested to target CXCR4 dimers. [17]

Thereafter, Nbs targeting CXCR7, also known to bind CXCL12, were identified. CXCR7, an atypical chemokine receptor now referred to as ACKR3, does not signal via G proteins but recruits β-arrestin, contributing to oncogenic signaling. Nbs targeting CXCR7 possess antagonistic properties inhibiting CXCL12 binding and recruitment of β-arrestin. [19] Biparatopic Nbs, targeting the N-terminus and extracellular loops of CXCR7 showed enhanced potencies. Extending these biparatopic Nbs with a Nb targeting albumin resulted in Nbs with increased half-life *in vivo*. These trivalent Nbs reduced tumor growth in a patient-derived, CXCR7-expressing head and neck cancer xenograft model, through inhibition of angiogenesis. [19] Cancer types showing high expression of CXCR7 might thus be effectively targeted by CXCR7 Nbs.

Highly potent and selective Nbs targeting CXCR2 have also been identified. Two distinct classes of CXCR2-targeting Nbs were described, one targeting the N-terminus and other ECL2. [20] The first class, which showed high affinity for CXCR2, only partially inhibited CXCL12 binding, while

the second showed lower affinity and more efficaciously inhibited chemokine-induced CXCR2 function. As previously shown for CXCR4 and CXCR7, the biparatopic Nbs were superior over their monovalent and bivalent counterparts. Interestingly, both monovalent as well as biparatopic Nbs displayed inverse agonistic properties. Using mutant and chimeric receptors, Bradley et al [20] convincingly demonstrated that the biparatopic Nbs bind CXCR2 dimers.

Altogether, Nbs targeting chemokine receptors display high affinity and specificity for their target. Moreover, their modular structure allows the generation of multivalent Nb formats with improved potencies, bispecific targeting or increased *in vivo* half-life. As such, they can be used to treat acute and chronic indications. Moreover, by conjugating fluorescent dyes to Nbs or radiolabelling them for use in positron emission tomography, Nbs are ideal probes for *in vivo* biomolecular imaging. Hence, Nbs targeting GPCRs are attractive research tools, and hold potential as diagnostics and therapeutics. [21]

Nanobodies Uncovered a Role of Hippocampal mGlu2 Receptors in Context Fear Consolidation

Dr. Jean-Philippe Pin

Jean-Philippe Pin (Université de Montpellier, CNRS, INSERM) started his talk by pointing out that, up to now, antibodies with therapeutic action targeting GPCRs act as antagonists, preventing endogenous ligand binding on the receptor. In many cases, therapeutic effects are expected upon activation of a GPCRs, but agonists often trigger receptor desensitization or even internalization, leading to a lack of effect after a short period of agonist application. The development of positive allosteric modulators (PAMs) is therefore viewed as the best alternative to agonists for positive action on GPCRs.

The aim of Dr. Pin's group was to examine whether antibodies with positive allosteric modulation could be identified. He presented his laboratory's work on metabotropic glutamate receptors (mGluRs) since these GPCR subfamily have a large extracellular domain undergoing major conformational changes upon activation, offering a way to stabilize an active state with an antibody. [22] These receptors are of interest for many therapeutic applications, including neurologic and psychiatric disorders. They are also involved in cancer development when ectopically expressed at the periphery. Therefore, Pin's lab is searching for single-chain Nbs because of their small size, ability to target cavities, and the possibility to manipulate their sequence. In their studies, cells expressing mGluR were injected into llamas, and a phage display library was generated after amplifying the VHH encoding sequences. Nbs that specifically recognized one mGluR subtype among the eight mGluRs, were identified. Two of these Nbs act as PAMs on this mGluR. One of them potentiates agonist actions on the activated mGluR. This PAM enhances the inhibitory action of the orthosteric mGluR agonist, at mossy fiber terminals in the CA3 region of hippocampal slices. It also impairs contextual fear memory, demonstrating its usefulness for the pharmacological control of mGluRs *in vivo*. These data also highlight the potential of developing antibodies with allosteric actions on GPCRs to better define their roles *in vivo*.

Generation of Antagonist Nanobodies for GPCRs by Genetic Immunization

Dr. Marc Parmentier

Generating antibodies recognizing the native conformation of GPCRs is usually regarded as difficult. In order to complement the tools available for receptors of interest, Marc Parmentier (Université Libre de Bruxelles) and his group generated in llamas monovalent single chain antibodies, or Nbs targeting the human chemokine CXCR4, the chemerin CMKLR1, and the vasoactive intestinal peptide VPAC1 receptors. As described by Dr Parmentier, a set of Nbs targeting CXCR4 was developed, following immunization of llamas with CHO-K1 cells expressing

the receptor or membrane preparations made from these cells. A phage display library was constructed in the pMES4 phagemid, and selection of relevant clones was performed by panning using viral pseudo-particles (VLP) containing CXCR4. The resulting clones were screened by fluorescence-activated cell sorting (FACS) on CXCR4-expressing CHO-K1 cells. Following labeling with DyLight 650, a set of Nbs bound to CXCR4 with a Kd in the range of 23 to 66 nM. Several Nbs were characterized as antagonists in a Ca^{2+} mobilization assay and in a chemotaxis assay involving human T lymphocytes. Some Nbs were also expressed in *Pichia pastoris* in order to increase the stability of CXCR4 produced in this system. [23]

For raising Nbs targeting CMKLR1, which is the main receptor for the leukocyte chemoattractant protein chemerin, llamas were immunized with the eukaryotic expression plasmid pVAX1 encoding human CMKLR1. Plasmid DNA (1-2 mg) was injected five times intradermally with a Dermojet device, and the llamas were boosted once with a camel cell line (DUBCA) expressing the receptor. A phage display library was constructed in the pXAP100 phagemid, and panning was performed either with VLPs or CHO-K1 cells expressing CMKLR1. Following screening by FACS on CHO-K1 cells expressing the receptor, Dr Parmentier's group identified two different Nbs targeting CMKLR1 (CA4910 and CA5183). These Nbs did not recognize the two other human receptors for chemerin, GPR1 and CCRL2, or mouse CMKLR1. In a saturation binding assay using Nbs labeled with DyLight 650, their respective KD was estimated to 100 and 280 nM. Competition binding assays also showed that the two Nbs share a common binding site, preventing generation of bispecific Nbs. They engineered, however, a bivalent CA4910 Nb exhibiting an increase in affinity. DyLight 650-labelled Nbs were shown to constitute tools as efficient as reference mouse mAb for immunofluorescence and FACS applications, including on human primary cells (monocyte-derived macrophages and dendritic cells). The binding site of the Nbs was shown to overlap with that of chemerin, and the Nbs inhibited binding of the C-terminal nonapeptide agonist (chemerin-9) much more efficiently than that of full-size chemerin. Both Nbs behaved as antagonists in an intracellular

calcium mobilization assay. Inhibition was complete when chemerin-9 was used as agonist, while partial when full-size chemerin was used. The bivalent Nb was much more efficient as an antagonist, and inhibited chemerin-induced chemotaxis of human monocyte-derived dendritic cells. [24] Altogether, the use of these Nbs and previously generated mouse mAbs support the existence of two independent binding sites for chemerin, one for the cystatin-like domain, the other for the C-terminal peptide that triggers receptor activation. The new Nbs also constitute interesting tools to study the role of the chemerin/CMKLR1 system in physiology and diseases.

Concluding remarks

Overall, the conference offered an interesting program with topics linking GPCRs and Abs from both fundamental and translational research perspectives. The talks nicely emphasized the recent advances in the technologies and strategies developed for: 1) the purification of GPCRs to be used as antigens in the immunization processes, and 2) the generation of new Abs using innovative *in vitro* and *in silico* approaches. Moreover, the successful development of various GPCR-selective Nbs with diverse pharmacological profiles undoubtedly shows the exciting progress in the field of GPCR Abs. This constitutes a sort of paradigm shift with the development of Nbs that are able not only to inhibit GPCRs (antagonists), but also to finely modulate their function either positively (full and partial agonists) or even allosterically (positive and negative allosteric modulators). Finally, the research on GPCR Abs is opening a new era on the utilization of Abs as a new generation of drugs for therapeutic applications in GPCR-linked diseases.

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